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ORIGINAL CONTRIBUTION

Consumption of polyphenol-rich *Morus alba* leaves extract attenuates early diabetic retinopathy: the underlying mechanism

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Abstract
Purpose Beneficial effects of white mulberry against diabetes mellitus have been reported. However, the molecular mechanisms of how white mulberry can attenuate diabetic retinopathy remain poorly understood. Here, the mechanism underlying the protective effect of *Morus alba* leaves ethanolic extract on oxidative stress, inflammation, apoptosis, and angiogenesis in diabetic retinopathy was investigated.
Methods Diabetes was induced by injection of streptozotocin. One week after, *M. alba* (100 mg/kg) was administrated to the rats daily for 16 weeks.
Results *Morus alba* extract showed high content of polyphenolics and free radical scavenging activity. Oral *M. alba* administration significantly attenuated hyperglycemia and weight loss, and decreased sorbitol, fructose, protein kinase C, pro-inflammatory cytokines, and oxidative stress markers in retinas of the diabetic rats. Moreover, *M. alba* produced marked down-regulation of caspase-3 and Bax, with concomitant up-regulation of Bcl-2 in the diabetic retinas. *M. alba* also reduced the expression of VEGF in the retina.
Conclusion These results indicate that *M. alba* has protective effect on diabetic retinopathy with possible mechanisms of inhibiting hyperglycemia-induced oxidative stress, apoptosis, inflammation, polyol pathway activation, and VEGF expression in the retina.

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Keywords Mulberry · Retina · Diabetes · Oxidative stress · Apoptosis

Introduction

Diabetic retinopathy (DR) is the most common diabetic eye complication and a well-known cause of blindness in working-age population [1]. It has been estimated that roughly 34.6 % of all diabetic patients have some forms of DR [2]. DR represents a spectrum of disease, ranging from patients who have diabetes but no evidence of DR through mild, moderate, and severe stages of nonproliferative DR and progressing to proliferative DR (PDR) [3]. The pathophysiology of DR is a multifactorial process. It involves complex interactions between hyperglycemia and oxidative stress [4]. The retina is vulnerable to reactive oxygen species (ROS) and lipid peroxidation because of its rich content of polyunsaturated lipid membranes. The hyperglycemia-induced oxidative stress induces retinal basement membrane thickening, a hallmark of microangiopathy, and increased retinal vascular permeability, perhaps leading to macular edema which correlates with vision loss in diabetic patients [5, 6].

In addition to triggering oxidative stress, hyperglycemia is involved in the pathogenesis of DR via multiple mechanisms such as increased activation of protein kinase C (PKC) [7], aldose reductase (AR) [8], and elevated nonenzymatic glycoxidation and glycation of proteins [9]. The β-isoform of PKC is considered as a major mediator of vascular endothelial growth factor (VEGF)-induced blood–retinal barrier (BRB) disruption and retinal neovascularization [10]. Intracellular accumulation of sorbitol resulted from increased AR activity might contribute to the breakdown of BRB in DR patients [11]. Moreover, accumulated advanced glycation end products (AGEs) in the

vascular wall have been reported to stimulate pro-inflammatory reaction and BRB breakdown in diabetes [12].

Many of the hyperglycemia-induced pathways merge to activate nuclear factor kappa B (NF-κB), with subsequent release of pro-inflammatory cytokines and oxidative stress, and finally lead to apoptosis [13]. Tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) are well-known representative inflammatory cytokines associated with the pathogenesis of DR. Through NF-κB activation, hyperglycemia-induced production of IL-1β and TNF-α results in apoptosis of endothelial cells and loss of retinal microvascular cells [14]. Thus, modulation of hyperglycemia-induced oxidative stress and inflammation might represent an important strategy for the treatment for DR.

Medicinal plants have been reported to be useful source of biologically active substances, including antioxidants and anticarcinogens [15]. White mulberry (*Morus alba* L., Moraceae) is a deciduous tree widely cultivated in subtropical, tropical, and moderate environments [16]. Mulberry is cultivated for fruit production [17], and its foliage is traditionally used as feed for silk worms [18]. Several recent studies have shown antioxidant, anti-inflammatory, antiviral, hypolipidemic, anti-hyperglycemic, neuroprotective, anti-hypotensive, and cytotoxic activities of different species of *Morus* [19–21]. Due to the presence of phenols, coumarins, and flavonoids, the leaves of *M. alba* possess pharmacological importance [22]. Since the leaves of *M. alba* have been recommended in the literature as a remedy for diabetes treatment, this study was carried out to explore its potential in management of experimentally induced DR in rats. This investigation could promote an understanding of its protective mechanism against diabetes-associated retinopathy, especially to the modulation of oxidative stress, inflammation, and apoptosis.

Materials and methods

Chemicals

Streptozotocin (STZ), reduced glutathione (GSH), pyrogallol, 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH), Folin–Ciocalteu reagent, gallic acid, rutin, thiobarbituric acid (TBA), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 1,1,3,3 tetramethoxypropane, sodium dodecyl sulfate (SDS), and 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Sigma (USA). All other chemicals were of analytical grade and supplied by standard commercial sources.

Preparation of *M. alba* leaves extract

M. alba leaves were collected from Beni-Suef governorate (Egypt), during the period from March to June 2013. The leaves were identified and authenticated by experts from Botany Department, Faculty of Science, Beni-Suef University (Egypt), and voucher samples were deposited at the Department of Botany, Faculty of Science, Beni-Suef University. The fourth and fifth leaves were plucked from the apex of healthy plants, washed thoroughly under running tap water, shade-dried for 5 days, and eventually ground to a fine powder in an electric grinder. The powdered plant material was extracted by maceration with 90 % ethanol for 72 h in ambient temperature. The extract was filtered with Whatman filter paper No 1, and filtrates were evaporated to dryness under reduced pressure in a rotary evaporator. The residual extract was used for the study.

Determination of total phenolics and flavonoids contents

Total phenolic content in the leaves extract of *M. alba* was determined according to the method of Waterman and Mole [23], using Folin–Ciocalteu reagent and gallic acid as a standard phenolic compound. Briefly, 200 µl of the extract solution was mixed with 1 ml of Folin–Ciocalteu reagent. After 5 min, 800 µl of sodium carbonate (75 g/L) was added and then incubated for 2 h at room temperature. The absorbance was measured at 760 nm.

Total flavonoids content was performed according to the method of Jia et al. [24] after slight modifications. The extract solution was mixed with 200 µl of 5 % sodium nitrite and incubated for 5 min at room temperature. 150 µl of 10 % aluminum chloride was added and finally mixed with 1 M sodium hydroxide. The absorbance was measured at 510 nm, and rutin was used as a standard flavonoid.

DPPH radical scavenging activity

A methanolic solution of DPPH was prepared and mixed with the extract solution with the ratio of 8:1. The mixture was shaken and incubated for 30 min at room temperature protected from light. The absorbance was measured at 517 nm [25]. Ascorbic acid was used as a standard antioxidant.

$ABTS^{\bullet+}$ radical scavenging activity

$ABTS^{\bullet+}$ radical scavenging capacity was determined according to the method of Re et al. [26]. $ABTS^{\bullet+}$ was prepared by reacting 2 mM ABTS in water with 2.45 mM potassium persulfate and was stored in the dark for 2 h at room temperature. The $ABTS^{\bullet+}$ was diluted in 0.1 M sodium phosphate buffer (pH 7.4) and mixed with the extract solution at the ratio of 1:3. The reaction mixture was incubated for 30 min at room temperature, and absorbance was measured at 730 nm. Ascorbic acid was used as a standard antioxidant.

Experimental animals

Adult male Wistar rats (*Rattus norvegicus*) weighing between 130 and 180 g were obtained from animal house of the National Institute of Opthalmology, El-Giza, Egypt. Rats were housed in standard cages at normal atmospheric temperature (25 ± 2 °C) and normal 12-h light/dark cycle. They were given access of water ad libitum and supplied daily with standard pellet diet of known composition (8.0 % moisture, 20.8 % crude protein, 4.8 % crude fat, 5.0 % crude ash, 37.2 % nonfiber carbohydrate, 3.2 % crude fiber, and vitamins and minerals adequate to meet the nutritional needs of rat). All animals were kept under observation before the onset of the experiment to exclude any intercurrent infection. All animal procedures were approved by the Institutional Ethics Committee of Beni-Suef University (Egypt).

Induction of experimental diabetes and animal grouping

Experimental diabetes mellitus was induced by a single intraperitoneal injection of freshly prepared solution of STZ (45 mg/kg body weight) in 0.1 M citrate buffer, pH 4.5 [27]. After 7 days of STZ administration, hyperglycemia was verified and rats having blood glucose levels ≥200 mg/dl were selected for the experiment.

Twenty-four rats were randomly divided into four equal groups, each consisting of six ($N = 6$) animals as follows:

Group 1: Normal control rats.
Group 2: Normal rats received 100 mg/kg/day *M. alba* extract dissolved in distilled water [28] by oral gavage for 16 weeks.
Group 3: Diabetic control rats.
Group 4: Diabetic rats received 100 mg/kg/day *M. alba* extract dissolved in distilled water [28] by oral gavage for 16 weeks.

Morus alba dose was balanced consistently as indicated by any change in the body weight to keep up comparable dosage over the entire period of study.

Samples collection and preparation

At the end of the experiment, overnight-fasted rats were euthanized by decapitation under mild ether anesthesia. Blood samples were collected to separate serum, and collected sera were stored at −20 °C until analyzed. The eye globes were quickly excised, and retinas were dissected and rinsed with ice-cold saline. Retina samples were homogenized in prechilled 0.2 M potassium phosphate buffer, pH 7.0, and used for assaying lipid peroxidation and antioxidant defenses. Some samples were kept frozen at −80 °C for Western blotting analysis. Other samples of the retina homogenized in 6 % (wt/vol) ice-cold perchloric acid, neutralized with potassium carbonate, were used to determine sorbitol and fructose concentrations.

Biochemical assays

Oral glucose tolerance test (OGTT)

On the day before killing, blood samples were obtained from lateral tail vein of control and diabetic rats deprived of food overnight. Successive blood samples were then collected at 30, 60, 90, and 120 min following the administration of 3 g/kg body weight glucose solution. Blood samples were left to coagulate and centrifuged for serum separation. Serum glucose concentration was determined according to the method of Trinder [29], using reagent kit purchased from bioMerieux chemicals (France).

Determination of insulin, fructosamine, and glycosylated hemoglobin (HBA1c)

Serum levels of insulin were determined using specific ELISA kits purchased from R&D systems (USA) following the manufacturer's instructions. Serum fructosamine levels were determined according to the method of Baker et al. [30] using reagent kit purchased from Spinreact Company (Spain). A blood sample from each rat was collected on ethylenediaminetetraacetic acid solution and used for the estimation of HBA1c % according to the method of Abraham et al. [31] using reagent kits purchased from Stanbio Company (Texas, USA).

Determination of sorbitol and fructose

Sorbitol and fructose concentrations were determined in the retinal homogenates following the method of Clements et al. [32] and Foreman et al. [33], respectively.

Determination of oxidative stress and antioxidant status

Lipid peroxidation was determined in retinal homogenates by measuring malondialdehyde (MDA) following the method of Preuss et al. [34]. GSH content and the activity of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) were assayed according to the methods of Beutler et al. [35], Cohen et al. [36], Marklund and Marklund [37], and Matkovics et al. [38], respectively.

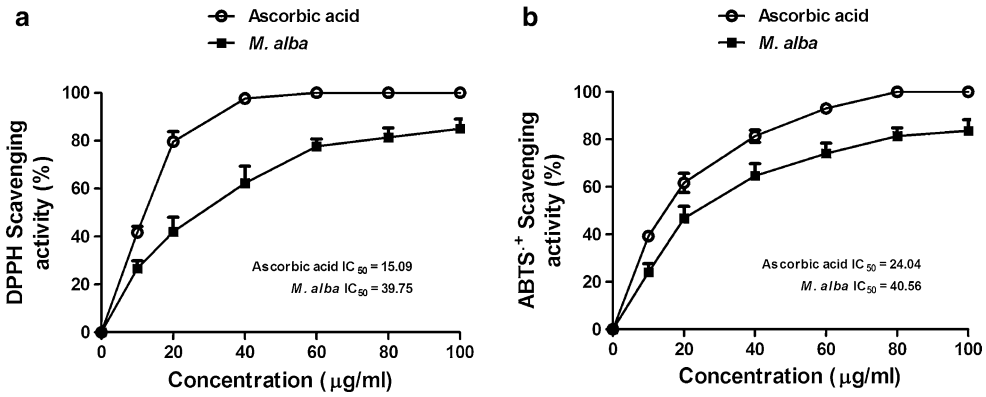

Fig. 1 DPPH (**a**) and ABTS (**b**) radical scavenging activity of *M. alba*. Data are the mean values of triplicate and expressed as mean ± SEM

Determination of TNF-α, IL-1β, and PKCβ

The levels of TNF-α, IL-1β, and PKCβ1 were determined in retina homogenates using specific ELISA kits (R&D systems) following the manufacturer's instructions. The concentrations of assayed parameters were measured spectrophotometrically at 450 nm. Standard curves were constructed by using standard TNF-α, IL-1β, and PKCβ1, and concentrations of the unknown samples were determined from the standard plots.

Western blotting analysis

The frozen retinas were homogenized in ice-cold lysis buffer. The samples were centrifuged at 10,000 g for 10 min to remove the insoluble material. Protein concentrations were determined according to the method of Bradford. Equal amounts of proteins were electrophoresed using 10 % SDS polyacrylamide gel electrophoresis and electro-transferred to nitrocellulose membrane. The membranes were blocked in 5 % w/v skimmed milk powder in phosphate-buffered saline (PBS)/Tween 20 (PBST) for 1 h at room temperature. The membranes were incubated with antibodies for VEGF, Bax, Bcl2, activated caspase-3, and β-actin (Santa Cruz Biotechnology, USA) diluted 1:1000 in blocking buffer. After washing, the membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature, washed, and then developed. The optical densities were quantified with ImageJ analysis software, normalized to β-actin and presented as % of control.

Statistical analysis

Data were analyzed using GraphPad Prism 5 software, and all statistical comparisons were made by means of the one-way ANOVA test followed by Tukey's test post hoc analysis. Results were articulated as mean ± standard error (SEM), and a P value <0.05 was considered significant.

Results

Total phenolic and flavonoid contents and antioxidant activity of *M. alba*

The amount of total phenolics in *M. alba* leaves 90 % (v/v) ethanol extract was 67.66 ± 2.92 mg gallic acid equivalent/g dry extract, and the recorded total flavonoids were 39.24 ± 1.18 mg rutin equivalent/g dry extract.

Results for the radical scavenging and antioxidant activity of *M. alba* leaves extract are represented in Fig. 1. The extract showed radical scavenging activity against DPPH and $ABTS^{\bullet+}$.

Morus alba represses body weight loss and hyperglycemia in diabetic rats

Data represented in Fig. 2a show the body weight changes after 16 weeks of treatment. STZ-induced diabetic rats exhibited significant ($P < 0.001$) body weight loss (−40.21 ± 7.91 g) when compared with the control rats (56.65 ± 10.02 g). Treatment of the diabetic rats with *M. alba* significantly ($P < 0.001$) prevented body weight loss and the rats recorded positively changed body weight (50.88 ± 6.03 g).

OGTT of STZ-induced diabetic rats showed significant elevation in blood glucose levels at fasting and at 30, 60, 90, and 120 min after oral glucose loading when compared with the control rats (Fig. 2b). Oral supplementation of *M. alba* extract to STZ-induced diabetic rats significantly ameliorated the blood glucose levels at all points of the OGTT. The OGTT areas under curve (AUCs) analysis showed a significant ($P < 0.001$) increase in STZ diabetic rats when compared with the control group. Treatment of the diabetic rats with *M. alba* potentially ($P < 0.001$) decreased OGTT AUC when compared with the diabetic control rats. Healthy

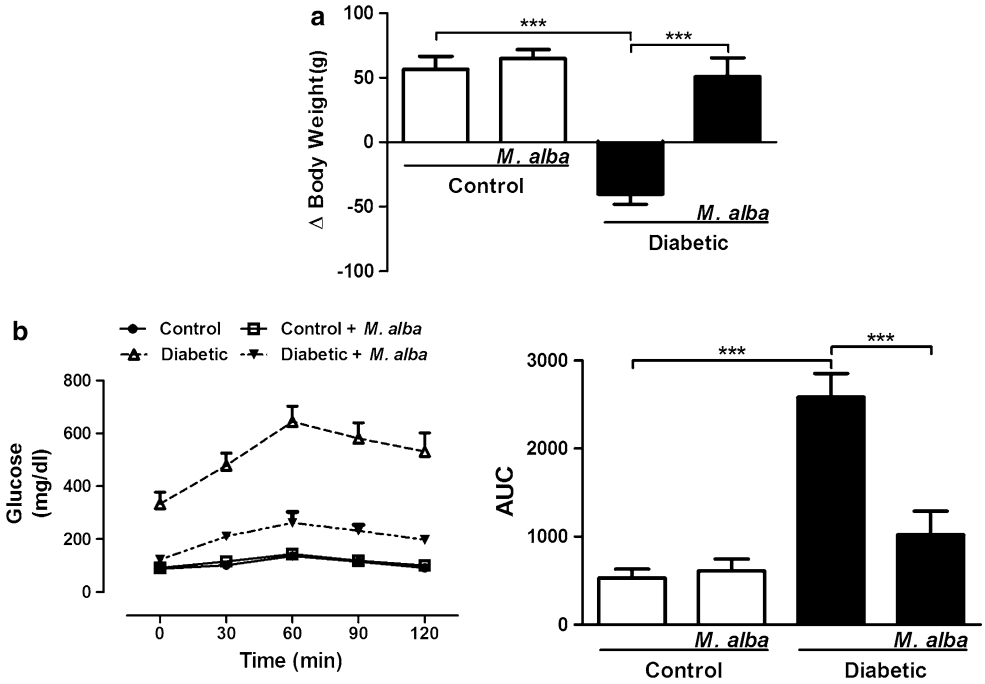

Fig. 2 Effect of *M. alba* administration on **a** body weight changes and **b** glucose tolerance in control and diabetic rats. Results are mean ± SEM ($N = 6$). ***$P < 0.001$. *OGTT* oral glucose tolerance test, *AUC* area under curve

Table 1 Effect of *M. alba* on serum insulin and fructosamine, and blood glycated hemoglobin levels

	Insulin (μIU/ml)	Fructosamine (μmol/L)	HbA1c (%)
Control	23.28 ± 2.52	173.55 ± 19.33	5.76 ± 0.57
Control + *M. alba*	23.50 ± 2.01	184.58 ± 10.05	5.17 ± 0.73
Diabetic	6.537 ± 0.226***	502.12 ± 49.39***	20.23 ± 1.41***
Diabetic + *M. alba*	14.990 ± 1.105$^{\#}$	145.17 ± 15.11$^{\#\#\#}$	10.30 ± 0.51$^{\#\#\#}$
F-prob.	$P < 0.001$	$P < 0.001$	$P < 0.001$

Data are M ± SEM, ($N = 6$)

*** $P < 0.001$ versus control, and $^{\#}$ $P < 0.05$ and $^{\#\#\#}$ $P < 0.001$ versus diabetic

rats received 100 mg/kg/day *M. alba* leaves extract for 16 weeks showed nonsignificant ($P > 0.05$) changes in body weight and glucose tolerance.

Morus alba ameliorates insulin release and attenuates protein glycation

Data summarized in Table 1 show the effect of *M. alba* on serum insulin and fructosamine levels, and HbA1c %. Serum insulin level was significantly ($P < 0.001$) decreased in STZ-induced diabetic rats compared to the control rats. Oral treatment of the STZ-induced diabetic rats with *M. alba* markedly ameliorated serum insulin levels. Conversely, diabetic rats exhibited significant ($P < 0.001$) increase in serum fructosamine levels and blood HbA1c % when compared with either the control or *M. alba*-treated diabetic rats. Oral supplementation of *M. alba* produced a significant ($P < 0.001$) decrease in serum fructosamine and blood HbA1c % in diabetic rats, with no recorded effect on normal rats.

Morus alba decreases the activity of the polyol pathway in retina of diabetic rats

To test the effect of *M. alba* on hyperglycemia-induced activation of the polyol pathway, sorbitol and fructose levels were determined in the retinal homogenates. Fructose levels showed a significant ($P < 0.001$) increase in retina of the STZ-induced diabetic rats (4746.86 ± 442.23 nmol/100 mg) when compared with the control group (591.23 ± 59.38 nmol/100 mg), as represented in Fig. 3a. Oral supplementation of *M. alba* leaf extract significantly ($P < 0.01$) decreased fructose level in the retina of diabetic rats (2979.40 ± 96.73 nmol/100 mg). Similarly, retinal content of sorbitol was significantly ($P < 0.001$) elevated in diabetic rats (90.61 ± 4.51 nmol/g) compared to the control group (29.97 ± 0.47 nmol/g). Treatment of the STZ-induced diabetic rats with *M. alba* produced a significant ($P < 0.01$) decrease in retinal sorbitol (69.22 ± 6.74 nmol/g) concentration (Fig. 3b). Fructose and sorbitol levels were nonsignificantly ($P > 0.05$) affected in

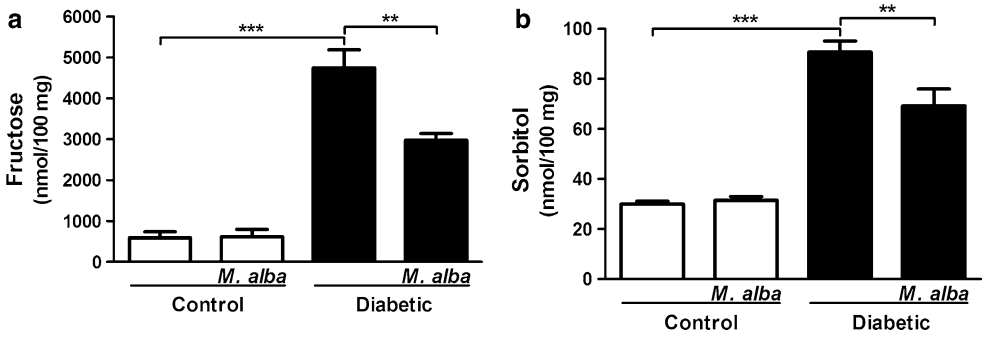

Fig. 3 Effect of *M. alba* on **a** fructose and **b** sorbitol levels in retina of control and STZ-induced diabetic rats. Results are mean ± SEM ($N = 6$). $^{**}P < 0.01$; $^{***}P < 0.001$

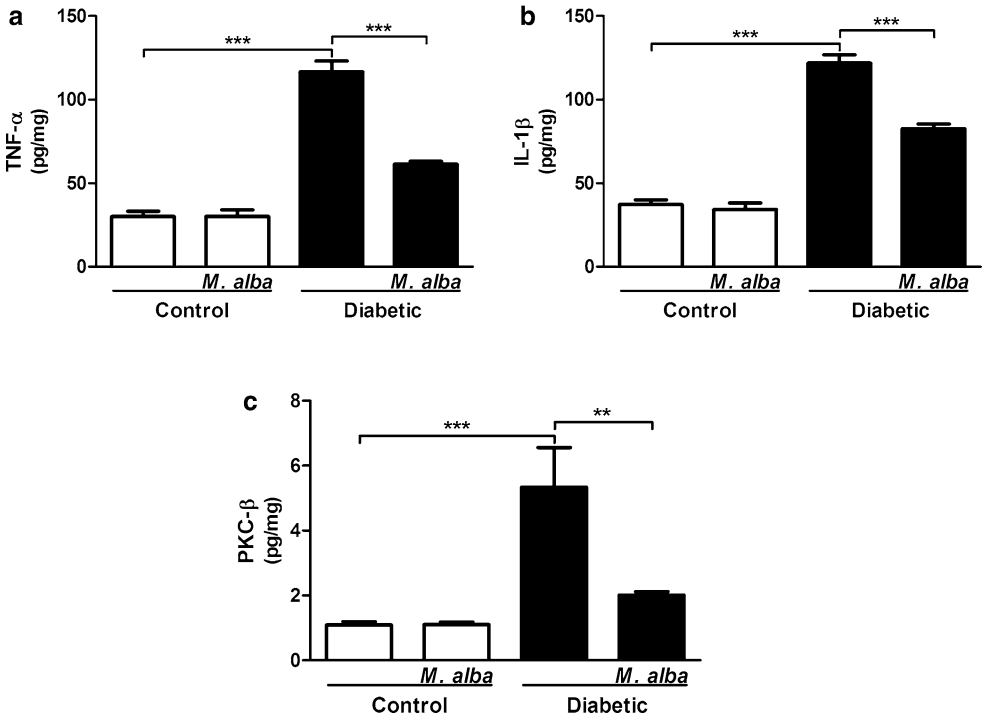

Fig. 4 Effect of *M. alba* on **a** TNF-α, **b** IL-1β, and **c** PKCβ levels in retina of control and STZ-induced diabetic rats. Results are mean ± SEM ($N = 6$). $^{**}P < 0.01$; $^{***}P < 0.001$. *TNF-α* tumor necrosis factor *alpha*, *IL-1β* interleukin 1 *beta*, *PKCβ* protein kinase C *beta*

M. alba-supplemented rats when compared with the control group.

Morus alba reduces inflammation and PKCβ in retina of diabetic rats

The levels of TNF-α (Fig. 4a) and IL-1β (Fig. 4b) in the retina of STZ-induced diabetic rats showed significant ($P < 0.001$) increase when compared with the corresponding normal control group. Oral supplementation of the diabetic rats with *M. alba* produced significant ($P < 0.001$) decrease in the levels of both TNF-α and IL-1β in the retina.

More or less similar, the levels of PKCβ were significantly ($P < 0.001$) increased in the retina of STZ-induced diabetic rats compared to the control group. Treatment of the diabetic rats with *M. alba* leaf extract significantly ($P < 0.01$) ameliorated retinal content of PKCβ, as represented in Fig. 4c. Of note, *M. alba* supplementation produced a nonsignificant ($P > 0.05$) effect on TNF-α, IL-1β, and PKCβ levels in retina of the normal rats.

Morus alba attenuates hyperglycemia-induced oxidative stress in retina of diabetic rats

Concerning lipid peroxidation, STZ-induced diabetic rats exhibited significantly ($P < 0.001$) increased MDA levels in retina (84.07 ± 1.49 nmol/100 mg) as compared to their respective normal controls (40.81 ± 1.01 nmol/100 mg), as shown in Fig. 5a. Treatment of the STZ-induced diabetic rats with *M. alba* extract markedly ($P < 0.001$) decreased retinal MDA content (64.08 ± 2.63 nmol/100 mg).

On the contrary, STZ administration produced a significant ($P < 0.01$) decrease in GSH content in retina of diabetic rats (1.95 ± 0.51 nmol/100 mg) when compared with the normal control group (6.09 ± 0.64 nmol/100 mg). Oral treatment of the diabetic rats with *M. alba* extract

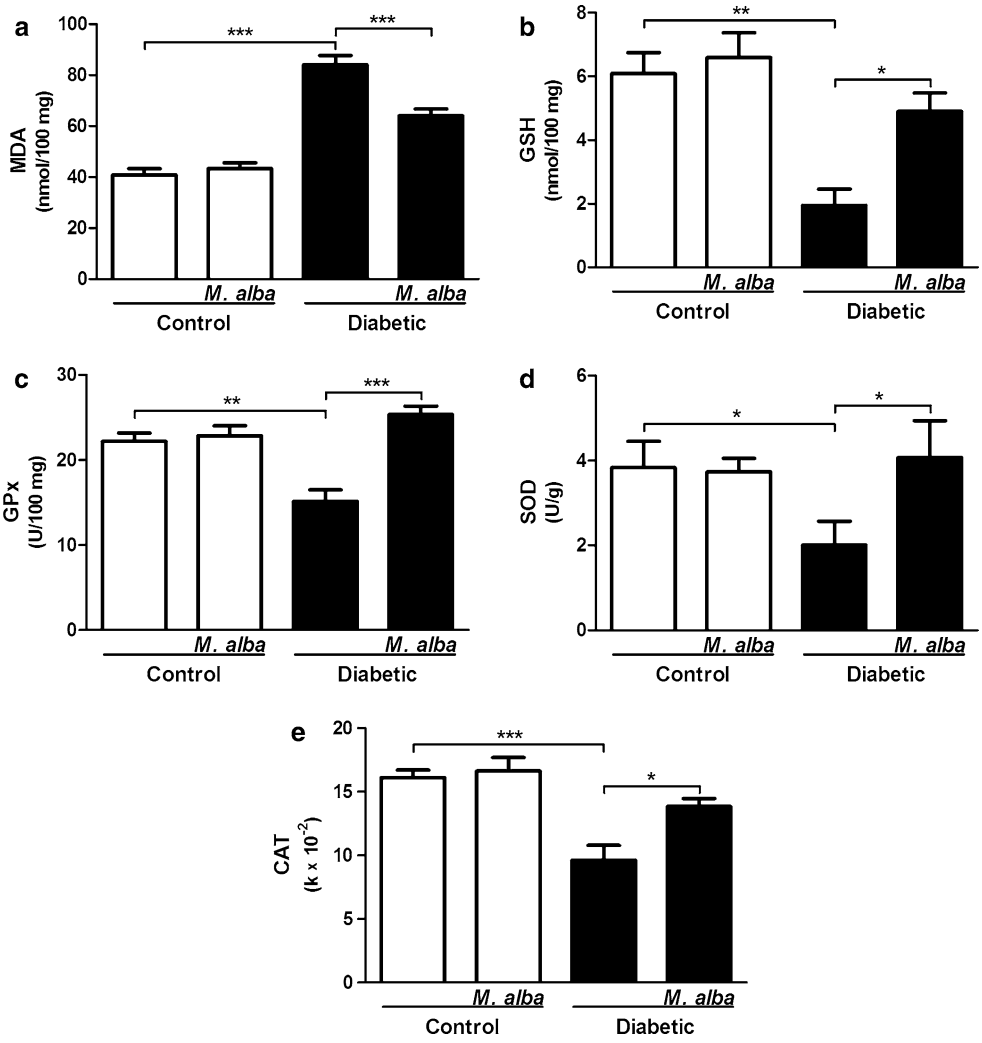

Fig. 5 Effect of *M. alba* on oxidative stress and antioxidant defense system parameters in retina of control and STZ-induced diabetic rats. Results are mean ± SEM ($N = 6$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. *MDA* malondialdehyde, *GSH* glutathione, *GPx* glutathione peroxidase, *SOD* superoxide dismutase, *CAT* catalase

significantly ($P < 0.05$) ameliorated retinal GSH content (4.90 ± 0.58 nmol/100 mg), as depicted in Fig. 5b.

GPx activity showed a similar pattern where it was significantly ($P < 0.01$) declined in the retina of STZ-induced diabetic rats (15.17 ± 1.32 U/100 mg) compared to the control group (22.22 ± 0.97 U/100 mg), as represented in Fig. 5c. Similarly, the activities of retinal SOD and CAT showed a significant decrease in STZ-induced diabetic rats (Fig. 5d, e). On the other hand, treatment of the diabetic rats with *M. alba* markedly increased the activities of GPx ($P < 0.001$), SOD ($P < 0.05$), and CAT ($P < 0.05$) in the retina. Retina of *M. alba*-supplemented normal rats showed nonsignificant changes in lipid peroxidation and antioxidant defenses.

Morus alba prevents apoptosis and angiogenesis in retina of diabetic rats

Western blotting analysis of the apoptosis proteins showed significant ($P < 0.001$) increase in protein levels of activated caspase-3 (Fig. 6a) and Bax (Fig. 6b) in retinas of STZ-induced diabetic rats. *M. alba* oral supplementation produced significant ($P < 0.001$) decrease in protein levels of activated caspase-3 and Bax in retina of the diabetic rats. In opposite, protein levels of the anti-apoptotic protein Bcl-2 showed a significant ($P < 0.001$) decrease in retina of STZ-induced diabetic rats and markedly ($P < 0.001$) increased following treatment with *M. alba* leaf extract (Fig. 6c).

Data represented in Fig. 6d show the effect of STZ-induced diabetes and treatment with *M. alba* on the protein expression levels of the angiogenesis marker VEGF in retina of rats. Diabetic rats exhibited significant ($P < 0.001$) increase in retinal protein levels of VEGF. On the other hand, treatment with *M. alba* leaf extract significantly ($P < 0.001$) attenuated diabetes-induced VEGF expression in retina of rats. *M. alba* exerted no effect on retinal activated caspase-3, Bax, Bcl-2, and VEGF when supplemented to normal rats.

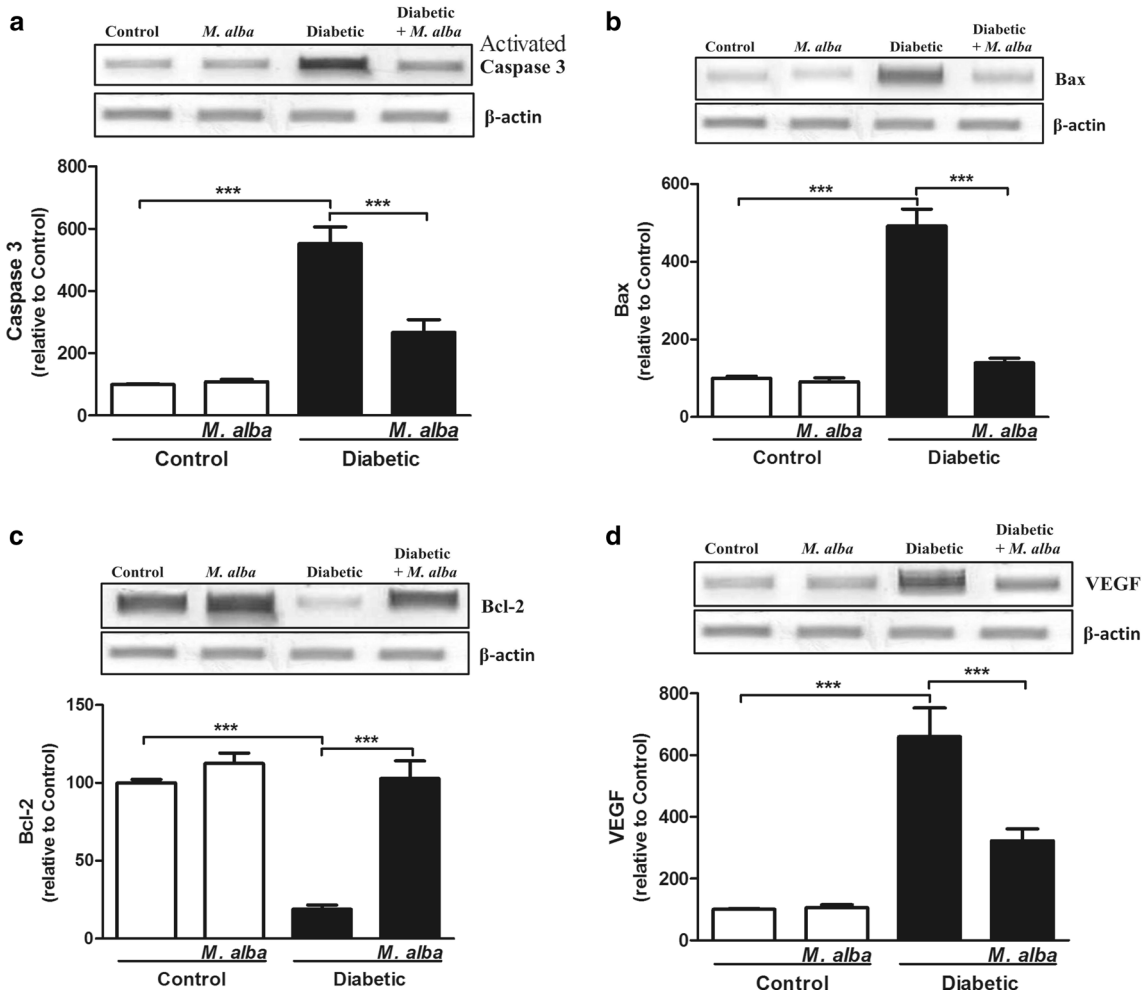

Fig. 6 Effect of *M. alba* on the expression of **a** activated caspase-3, **b** Bax, **c** Bcl-2, and **d** VEGF in retina of control and STZ-induced diabetic rats. Results are mean ± SEM ($N = 6$). ***$P < 0.001$

Discussion

DR is a common diabetes complication and is a leading cause of blindness in working-age population. Since clinical trials with pharmacologic agents that inhibited one of the specific pathways of DR showed disappointing results [39], inhibition of various pathways might represent an important strategy for the prevention of DR. Therefore, the current study was undertaken to evaluate the possible effectiveness of a polyphenol-rich *M. alba* leaves extract on hyperglycemia-induced oxidative stress, apoptosis, inflammation, and VEGF expression in retina of STZ-induced diabetic rats.

Under insulin deficiency and hyperglycemic conditions, the body provides itself energy by degrading proteins and lipids, which ultimately accounts for body weight loss [40]. Accordingly, STZ-induced diabetic rats in the present study showed significant hypoinsulinemia and weight loss. Treatment with *M. alba* leaves extract for 16 weeks attenuated hyperglycemia and its associated body weight loss, suggesting possible improvement in energy metabolism. These findings could be explained, at least in part, due to the insulinotropic effect of *M. alba*. In this context, Mohammadi and Naik [41] reported that *M. alba* increased serum insulin levels in diabetic rats through its ability to stimulate the spontaneous recovery of β-cells of the islets of Langerhans. In addition, the ameliorative effect of *M. alba* extract on blood glucose level seems to be mediated through other mechanisms. *M. alba* and some of its constituents have demonstrated an ability to inhibit hepatic gluconeogenesis by suppressing glucose 6-phosphatase activity [42], increase hexokinase, and glucose-6-phosphate dehydrogenase activity [43], and enhance hepatic glycogen synthesis secondary to β-glucosidase inhibitory activity [44]. The present findings are in agreement with several previous studies [28, 45, 46].

Oxidation of glucose is one of the mechanisms involved in the pathogenesis of diabetes complications [47]. It enhances glycation of hemoglobin and produces HbA1c [48]. The concentration of HbA1c is a good marker for diagnosis and prognosis of diabetes complications, and is strongly related to the risk of DR [49]. Hyperglycemia-triggered elevated HbA1c leads to red blood cell stiffening and decreased deformation capacity. As a result, both blood viscosity and shear stress at the endothelium of retinal vessel increase, leading to damage of the blood vessel integrity and pericytes loss [50]. Subsequently, the developed hypoxia results in compensatory expansion of retinal blood vessels to increase perfusion [51]. Reduction in HbA1c % in diabetic rats after 16-week *M. alba* supplementation indicates that *M. alba* has beneficial effects in attenuation of DR. In this context, the study conducted by Kowluru and Chan [52] reported that reduction of only one unit (~7 %) of HBA1c can reduce the risk of DR by over 30 %. Since insulin treatment of STZ diabetic rats significantly lowered the level of HbA1c [53], the insulinogenic effect of *M. alba* extract and the subsequently improved glycemic state account for its improved levels. The ability of *M. alba* to control the glycemic state was further evidenced by the lowered serum fructosamine level. Fructosamine is an early glycation end product results from the nonenzymatic reaction between glucose and amino acids [54]. It can be used to predict the concentration of AGEs and is an indicator of glycemic control over a 3 weeks or longer period [55]. In addition, the polyphenolic compounds, especially flavonoids, have the ability to interfere with protein glycation through scavenging free radicals. Here, we confirmed the presence of phenolics and flavonoids as well as the radical scavenging activity of *M. alba* leaves extract.

Many hyperglycemia-induced metabolic abnormalities such as increased oxidative stress are implicated in the pathogenesis of DR [52]. Chronic hyperglycemia has been reported to trigger oxidative stress either by direct generation of ROS or by altering the redox balance [48]. ROS is produced by multiple pathways including xanthine oxidase, the mitochondrial electron transport chain, and uncoupled nitric oxide synthases [56]. PKC activation, formation of AGEs, and polyol pathway can also contribute to oxidative stress by diminishing the activities of antioxidant enzymes [57]. Because of the highest uptake of oxygen and its high concentration of polyunsaturated fatty acids, retina is vulnerable to lipid peroxidative damage [6]. Several animal studies have demonstrated that hyperglycemia-induced oxidative stress is linked to the retinal capillary basement membrane thickening, which is an early abnormality of the microangiopathy seen in DR [6]. In addition, increased oxidative stress in diabetes mellitus is proposed to play central role in capillary cell apoptosis [58]. Thus, oxidative stress is a major contributor in the development of DR, and this makes it an important target for therapeutic strategies for this disease.

In the current study, retina of diabetic rats showed a significant increase in the lipid peroxidation marker, MDA, with concomitant declined GSH content and activity of SOD, CAT, and GPx. Our data are in agreement with the study of Soufi et al. [59] in which diabetic rats experienced chronic hyperglycemia with an increase in oxidative stress markers. *M. alba* supplementation markedly attenuated hyperglycemia-induced oxidative stress through preventing GSH depletion and enhancement of the enzymatic antioxidants. The antioxidant effects of *M. alba* were further confirmed by the in vitro DPPH and $ABTS^{\bullet+}$ radical scavenging assays. These effects could be directly linked to the rich polyphenolic constituents especially the flavonoids in *M. alba*. The leaves of mulberry contain high amounts of quercetin, quercetin 3-(6-malonylglucoside), rutin, oxyresveratrol, and 5,7-dihydroxycoumarin 7-methyl ether which are responsible for their antioxidant potential [60, 61].

Activation of PKC is another pathway implicated in the development of DR. In diabetes, elevated levels of diacylglycerol induced by hyperglycemia activate PKCβ [62]. PKC activation contributes to ROS production by increasing the activity of NADPH oxidase [63], increases the expression of VEGF [64], and decreases nitric oxide production in smooth muscle cells [65]. PKC is also implicated in NF-κB activation and thus connects hyperglycemia-induced oxidative stress to inflammation [66]. Because of the multiple effects of elevated PKC activation, it may be considered as a promising therapeutic target for DR. Increased activation of PKCβ occurs in retinas of diabetic animals and in endothelial cells exposed to high glucose (reviewed in Frank [67]). The elevated levels of PKCβ in retina of the diabetic rats in the present investigation provide additional evidence. Interestingly, retina of the diabetic rats received *M. alba* for 16 weeks showed decreased levels of PKCβ. Inhibition of PKC using general and specific inhibitors prevented retinal vascular permeability [62]. Therefore, attenuation of PKCβ seems to have a role in the protective mechanism of *M. alba* against DR.

Polyol pathway is one of the major pathways implicated in the development of DR. In this pathway, glucose is converted to sorbitol by the enzyme AR using NADPH as a cofactor. Sorbitol is further processed to fructose by the action of sorbitol dehydrogenase using NAD^+ as a hydrogen donor [68]. Diabetic rats in the present investigation showed significant increase in retinal levels of sorbitol and fructose, indicating activated polyol pathway. Increased sorbitol level during hyperglycemia occurs due to the flux of glucose through the polyol pathway [69]. AR is the rate-limiting enzyme in the polyol pathway [70] and could be considered as an attractive therapeutic target for DR. Therefore, the protective effects of pharmacological

inhibition and genetic deletion of AR have been examined in several studies. The specific AR inhibitor zoloperstat prevented ROS generation and retinal endothelial cell death [71]. Genetic deletion of AR protected diabetic mice against ROS production and retinal acellular capillaries [72]. The present data showed that treatment of the diabetic rats with *M. alba* attenuated hyperglycemia-induced sorbitol and fructose production, possibly through inhibition of AR. Recently, the AR inhibitory activity of *M. alba* and its flavonoid morusin was demonstrated by Rao et al. [73].

It is now increasingly appreciated that the pathogenesis of DR involves low-grade inflammation [74]. Several inflammatory cytokines are known to participate in the breakdown of BRB in diabetes. IL-1β and TNF-α are the representative inflammatory cytokines associated with the pathogenesis of DR. Their level is increased in both the vitreous humor and serum of patients with PDR [75]. In the present study, the levels of IL-1β and TNF-α showed a significant increase in retina of diabetic rats, reflecting the degree of inflammation. In agreement with our findings, the levels of IL-1β were found to be increased in retinas from diabetic rats [76]. Also, the activity of caspase-1, a proteolytic enzyme involved in the production of IL-1β, is up-regulated in the retinas of diabetic patients [77]. TNF-α as well is involved in the loss of retinal microvascular cells in diabetic retina [14]. Oral supplementation of the diabetic rats with *M. alba* for 16 weeks potentially attenuated the production of IL-1β and TNF-α in the retina, confirming a potent anti-inflammatory activity.

In addition, *M. alba* proved a potent anti-apoptotic activity as evident by down-regulation of Bax and caspase-3 and up-regulation of Bcl-2 protein expression in the retina of diabetic rats. The induced apoptosis in retina of diabetic rats could be directly connected to the hyperglycemia-induced inflammation. Endothelial IL-1β overexpression, stimulated by high concentration of glucose, induces apoptosis of endothelial cells through NF-κB activation in vitro. In addition, IL-1β has been reported to accelerate apoptosis in retinal pericytes under high glucose conditions through activation of NF-κB [78]. Likewise, TNF-α is involved in the loss of microvascular cells in diabetic retina [14]. It disturbs expression and subcellular localization of the tight junction proteins, claudin-5 and ZO-1, in bovine retinal endothelial cells [79]. The pro-inflammatory and pro-apoptotic effects of IL-1β and TNF-α were further confirmed through knockout studies and pharmacological inhibition. In the IL-1β receptor knockout mice, diabetes-induced retinopathy was markedly attenuated at 7-month duration of diabetes [80]. In addition, inhibition of caspase-1 using minocycline decreased the degeneration of retinal capillaries in the treated animals [80]. Similarly, TNF-α knockout protected rat against diabetes-associated retinal apoptosis, leukostasis, and breakdown of BRB [81].

The anti-inflammatory effect of *M. alba* in the present study is in agreement with several investigations. Choi and Hwang [82] reported the anti-inflammatory effects of *M. alba* leaf extract in RAW264.7 macrophages. Oxyresveratrol, an active ingredient of *M. alba*, has been previously demonstrated to exert anti-inflammatory activity through inhibition of NF-κB activation, iNOS/NO production, and PGE2 synthesis [83]. More recently, Chen et al. [84] reported the anti-inflammatory effects of both *M. alba* and the active compound oxyresveratrol. Moreover, prenylated flavonoids from *M. alba* prevented the lipopolysaccharide-induced inflammatory response in macrophages [85]. Quercetin and rutin, a flavonol and its glycoside present in *M. alba* [60], have been reported to exert anti-inflammatory, antioxidant, and anti-apoptotic effects in STZ-induced diabetic rat retina [86, 87].

Oxidative stress and pro-inflammatory cytokine are implicated in VEGF up-regulation in the diabetic retina [57]. In addition to induction of apoptosis, IL-1β is known to increase the expression of VEGF in retinal endothelial cells [77]. VEGF is a potent vascular permeability factor, and studies demonstrated its up-regulation in neovascular eye diseases including DR [88]. In addition, increased levels of VEGF have been identified in ocular fluids of patients with PDR [89]. Accordingly, diabetic rats in the present investigation showed significant up-regulation of retinal VEGF protein levels. *M. alba* supplementation for 16 weeks alleviated VEGF expression levels in retina of the diabetic rats. These findings could be attributed to the anti-inflammatory and anti-angiogenic effects of *M. alba* leaves extract. A recent study conducted by Hong et al. [90] reported the anti-angiogenic effect of a herbal composition containing *M. alba*. More or less similar, administration of anti-VEGF antibodies to experimental animals attenuated high glucose-induced vascular hyperpermeability [91]. In addition, clinical trials using anti-VEGF therapy are displaying promising results against stages of DR [92]. Therefore, down-regulation of VEGF seems to participate in the protective mechanism of *M. alba* against hyperglycemia-induced DR. For achieving better results, additional research is required to elucidate the effect of *M. alba* extract on histopathological alterations of retina in diabetic animals.

In conclusion, the present study depicts that *M. alba* administration proved a potent anti-hyperglycemic effect. Since DR is triggered by a persistent increase in blood glucose levels, good glycemic control can reduce its development. *M. alba* has protective effect on DR with possible mechanisms of inhibiting hyperglycemia-induced oxidative stress, apoptosis, inflammation, polyol pathway activation, and VEGF expression in the retina of diabetic rats (summarized mechanistic pathways are presented in Fig. 7). Given the key role of oxidative stress in the progression of DR,

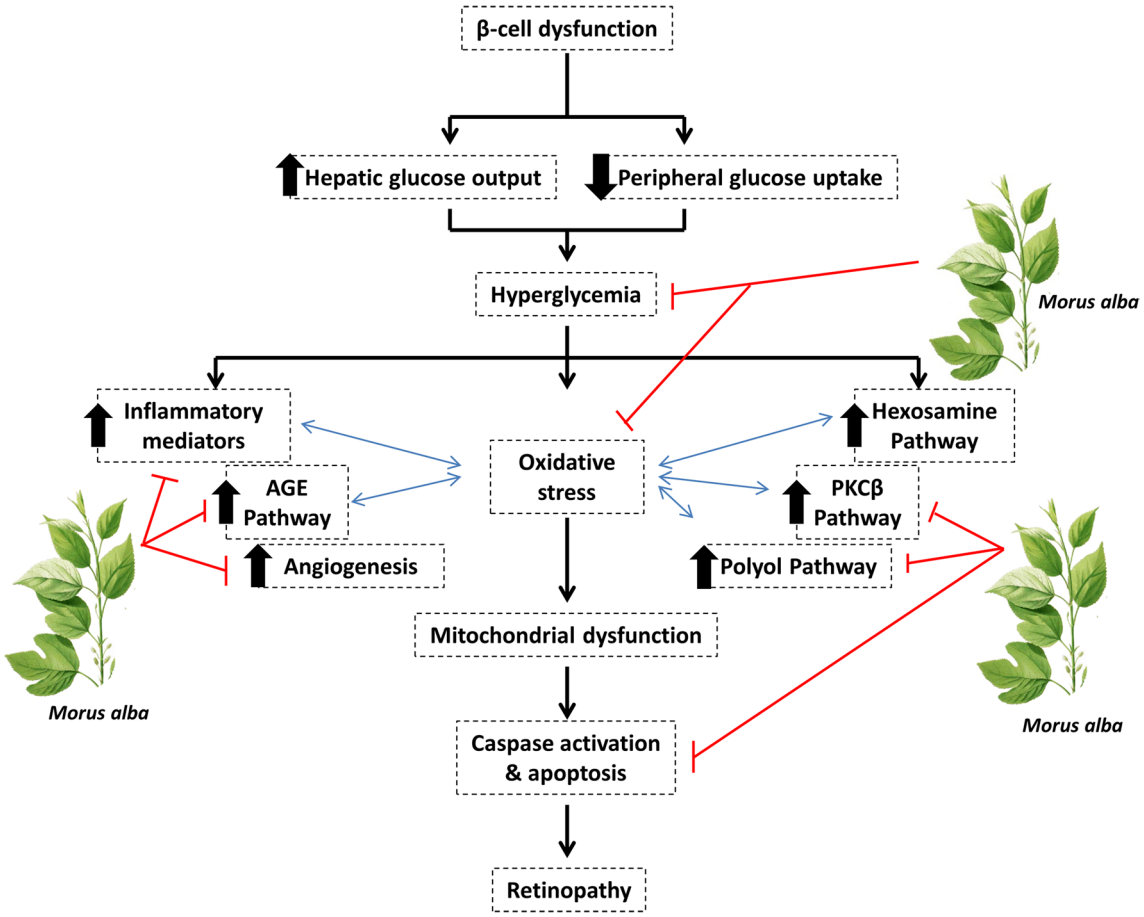

Fig. 7 A proposed schematic *diagram* for the mechanisms of the hyperglycemia-induced retinal damage and the alleviating effects of *M. alba*

the observed antioxidant and anti-diabetic properties of *M. alba* make it candidate as a therapeutic supplement to reduce DR.

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Compliance with ethical standards

Conflict of interest The authors have declared that no competing interests exist.

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